Guayusa (*Ilex guayusa* L.) new tea: phenolic and carotenoid composition and antioxidant capacity

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ABSTRACT

BACKGROUND: Guayusa (*Ilex guayusa* Loes) is an evergreen tree native of South America that grows particularly in the upper Amazon region of Ecuador. For its health benefits, it has been cultivated and consumed since ancient time by Amazon indigenous tribes.

RESULTS: A total of 14 phenolic compounds were identified and quantified. Chlorogenic acid and quercetin-3-*O*-hexose were the main representatives of the hydroxycinnamic acids and flavonols, respectively. Five carotenoids were identified, showing lutein the highest concentration. Guayusa leaves revealed high antioxidant capacity determined by two analytical methods: DPPH and ORAC. The industrial

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processing applied to the leaves modified the composition of bioactive compounds and antioxidant capacity of guayusa. In general, blanched guayusa retained the concentration of phenolic compounds and some carotenoids and similar antioxidant capacity as untreated green leaves. Oppositely, fermentation reduced the content of bioactive compounds and showed the lowest antioxidant capacity.

CONCLUSION: Therefore, blanched guayusa has potential for product development as functional ingredient in food industry.

Keywords: *Ilex guayusa*, phytochemical composition, antioxidant capacity, industrial processing

INTRODUCTION

Guayusa (*Ilex guayusa* Loes) is an evergreen tree native of South America that grows between Southern Colombia and Northern Peru, particularly in the upper Amazon region of Ecuador. The distribution of this species is from sea level to 2,000 m.[1] Guayusa is distantly related to the "Yerba Mate" or Mate" (*Ilex paraguariensis*), both plants being described as sources of caffeine. [2]

For its health benefits, such as alleviating diverse pains and preventing undesirable central nervous system effects, guayusa has been cultivated and consumed since ancient time by Amazon indigenous tribes, particularly Kichwa and Shuar, forming an part

importantly of their rituals and ceremonies.[2,3] The leaves are prepared as the tea, that is to say, as a hot infusion of the dried and minced leaves and twigs of *I. guayusa*, its flavor being naturally smooth and never bitter, with a rich and earthy aroma and slightly sweet finish. Nowadays, the consumption of guayusa is expanding, being marketed as infusion, energy drink and/or ingredient in other products in Ecuador, USA, China and Europe.[1, 4] This reflects the interest of consumers to incorporate new foods or new food ingredients with health promoting effects.

The major constituents of *Ilex* spp. are phenolic compounds.[5,6] These compounds are of great interest since they are attributed various beneficial biological effects; the most relevant is antioxidant activity, which is important in countering oxidative stress[7]. In addition, carotenoids have also been associated with numerous beneficial effects on human health: enhancement of the immune response and reduction of the risk of degenerative diseases such as cancer, cardiovascular diseases, cataract, and macular degeneration. The action of these compounds against diseases has been primarily attributed to their antioxidant capacity, but could also be due to other mechanisms, like the absorption of blue light in the retina or actions at the molecular level.[8] Thus, it is important to characterize the phenolic and carotenoid composition in order to correlate their benefits with human health. In this sense, the characterization of these bioactive compounds requires advanced analytical techniques. Concretely, reverse-phase liquid chromatography (RP-LC) is the most common method for the separation and analysis of phenolic compounds while UV-detection and mass spectrometry are the techniques commonly used for identification and quantification of phenolic compounds in plants.[9] Rapid Resolution Liquid Chromatography (RRLC) is employed for the characterization of carotenoids. [10]. Furthermore, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and the oxygen

radical absorption capacity (ORAC) have been frequently used to estimate antioxidant capacities in plants and foods.[11]

On the other hand, industrial processing of *I. guayusa* involves steps as blanching or fermentation which may cause some changes in the profile and concentration of their bioactive compounds (i.e. polyphenols and carotenoids) that could modify its biological activities. Blanching is a process of preheating the product by immersion in water or steam whose main objective is the inactivation of the enzymes present in foods.[12] Meanwhile, fermentation in guayusa manufacturing does not involve a microbial process, as in wine and beer, but chemical changes in their leaves catalyzed by endogenous enzymes.

The characterization of bioactive compounds like carotenoids and phenolics in edible native plants and the effect of processing on them is always a timely research topic.[13,14]

The interest in natural antioxidants found in plants has also increased due to the world-wide increase in using plant extracts as additives in food. Taking into account this consideration, the main objective of this study was to characterize for the first time the phenolic and carotenoid composition and antioxidant capacity of *I. guayusa*. Additionally, and in order to evaluate the effect of industrial processing on the composition of bioactive compounds of guayusa, the carotenoid and phenolic fraction as well as the antioxidant capacity of blanched and fermented guayusa were analyzed.

MATERIAL AND METHODS

Standards, Chemicals and solvents

The commercially available standards of phenolic compounds: gallic acid, 5-*O*-caffeolyl-quinic acid and quercetin 3-*O*-rutinoside were pursached from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Reagents 2,2-diphenyl-1-picrylhidracyl radical ($DPPH^{\cdot}$), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), monobasic sodium phosphate, dibasic sodium phosphate, Folin Ciocalteu's reagent and fluorescein (free acid) were obtained from Sigma–Aldrich (Taufkirchen, Germany) while 6-hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Fluka Chemika (Neu-Ulm, Switzerland). Meanwhile, formic acid, ethanol, methanol, hexane, acetone, dichloromethane and acetonitrile were all of analytical grade (Merck, Darmstadt, Germany). The chromatographic solvents were methanol, acetonitrile, ethyl acetate (HPLC grade, procured from Merck, Darmstadt, Germany). β-carotene and lutein were purchased from Sigma-Aldrich (Taufkirchen, Germany). Violaxanthin, α-carotene and lutein were isolated from natural sources by classical chromatographic techniques.[15]

Plant material

Leaves of fresh and processed guayusa (*Ilex guayusa*) were kindly provided by RUNA Foundation (Archidona, Napo, Ecuador). Guayusa leaves were collected in Pastaza, Ecuador. The collection and the storage of the material were realized under strict controlled conditions. Green leaves: fresh leaves just harvested; this material was stored at -20 ºC until freeze-dried. Blanched and fermented guayusa were carried out in the manufacturing plant of RUNA Foundation, according to standard protocols in this company. Then, leaves of blanched and fermented guayusa were freeze-dried. Finally, leaves of green and processed guayusa were crushed with a mortar.

Extraction procedure

For the Total Phenolics Content (TPC) and HPLC analysis, each sample (100 mg) was mixed with 1 mL of methanol/water (70:30, v/v) and was acidified with 1% of formic acid. Then, the samples were vortexed and sonicated in an ultrasonic bath for 60 min. The samples were kept at 4 ºC overnight and sonicated again for 60 min. A centrifugation (model EBA 21, Hettich Zentrifugen) step (9500 xg, 5 min) was used to separate the supernatant from the solid residue. This supernatant was filtered through a 0.45-µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 ºC before the analyses were performed.

For Rapid Resolution Liquid Chromatography (RRLC) analysis, 10 mg of homogenized freeze-dried powder from the samples were used for the extractions. The powder was gently mixed with 1 mL of the MiliQ-water and then vortexed and centrifuged to remove the aqueous phase at 18,000 × *g* for 3 min. Subsequently, 1 mL of extracting solvent (hexane/acetone, 1:1 v/v) was added, the mixture was vortexed and then centrifuged for 3 min at 18,000 × *g*. After recovering the colored fraction, a further 500 mL of extracting solvent was added, and the mixture was vortexed and finally spun as explained before. The pooled organic colored fractions were eventually evaporated to dryness in a vacuum concentrator at a temperature below 30◦C and stored under N_2 at −20ºC until analysis.

Total Phenolics Content (TPC)

The determination of the TPC using the colorimetric method of Slinkard and Singleton[16], based on the oxidation of the hydroxyl groups of phenols in basic media by the Folin-Ciocalteu reagent, was used. Briefly, an aliquot (0.5 mL) of the extracts, blank or standard was placed in a 15 mL tube, where the Folin–Ciocalteu reagent (2.5 mL) was added and the mixture was allowed to react for 2 min under continuous stirring

before a solution of sodium carbonate (75 g/L, 2 mL) was added and mixed well. The mixed was incubated at 50 °C during 15 min. The absorbance was then measured at 750 nm using a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The results are expressed as mg gallic acid equivalents (GAE)/g dried weight (DW), using a calibration curve over the range of 10 – 90 mg/L. This method is not specific for polyphenols because other reducing compounds will also be included in the quantification, but it is widely used for standardization between studies and species and also for comparison with other species. Therefore, we also used chromatographic and mass spectra analysis for the characterization of the polyphenols in guayusa samples.

Identification of phenolic compounds by HPLC–DAD–ESI/MS^n and quantification by HPLC–DAD.

The extraction and analysis of phenolic compounds were performed using the method of Gironés-Vilaplana et al.[17] In relation to the identification, it was carried out by HPLC–DAD–ESI/MS^n analyses, using an Agilent HPLC 1100 series model equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by Chem-Station software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface, and was controlled by LCMSD software (Agilent, version 4.1). The ionization conditions were 350 ºC and 4 kV, for capillary temperature and voltage, respectively. The nebulizer pressure and nitrogen flow rate were 65.0 psi and 11 L/min, respectively. The full-scan mass covered

the range of m/z from 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. The mass spectrometry data were acquired in the negative ionization mode. The MS^n was carried out in the automatic mode on the more abundant fragment ion in MS (n -1).

For the quantification, the HPLC-DAD system (Agilent 1220-Infinity LC) was used and the compounds separated in a Luna C18 column (25 cm x 0.46 cm, 5 μm particle size; Phenomenex, Macclesfield, UK). The individual phenolic compounds were tentatively identified following their characteristic UV-Visible spectra, order of elution (Retention time (Rt)), and comparison with standards previously established in the HPLC-DAD–ESI-MS^n method. Hydroxycynnamic acids were quantified using chlorogenic acid (5-*O*-caffeolyl-quinic acid) as standard at 330 nm and flavonols using rutin (quercetin 3-*O*-rutinoside) at 360 nm. All the samples were extracted in triplicate and injected three times. The results are expressed as mg/g DW.

Identification and quantification of carotenoid compounds by Rapid Resolution Liquid Chromatography (RRLC)

The analyses of carotenoids were carried out according to the method described by Stinco et al.[10] The extracts were saponified to eliminate the chlorophylls. The saponification conditions were: methanolic KOH (30 g/100 mL) for 1 h under dim light and at room temperature, after which they were washed with water to remove any trace of base. The colored dichloromethane extracts obtained were concentrated to dryness in a rotary evaporator at temperature below 30 °C and dissolved in 100 μL of ethyl acetate prior to their injection in the RRLC system. All the samples were extracted in triplicate. The identification was made by comparison of their chromatographic and UV–vis

spectroscopic characteristics with those of standards. Meanwhile, the quantification was carried out by external calibration from the areas of the chromatographic peaks obtained by DAD detection at 450 nm. The results are expressed as μg/g DW.

Antioxidant capacity

Antioxidant activity was determined by the DPPH$^{\cdot}$ and ORAC methods adapted to a microscale. DPPH$^{\cdot}$ test was realized according to the method described by Mena et al.,[18] where the antioxidant activity was evaluated by measuring the variation in absorbance at 515 nm after 50 min of reaction with DPPH$^{\cdot}$. The reaction was started by adding 2 μL of the corresponding diluted sample to the well containing 250 μL of DPPH$^{\cdot}$ dissolved in methanol. In relation to ORAC, it was performed according to Ou et al.[19] Briefly, the reaction was carried out at 37 ºC in 10 mM phosphate buffer (pH 7.4) and the final assay mixture (200 μL) contained fluorescein (1 μM), 2,2′-azobis(2-methyl-propionamidine)-dihydrochloride (250 mM) and antioxidant (Trolox [10 - 200 μM] or guayusa samples (methanol/water, 70:30 v/v) [at different concentrations]). DPPH$^{\cdot}$ and ORAC assays were performed using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite® M200 microplate reader (Tecan, Grödig, Austria). Assays were conducted in triplicate. The results are expressed as mmol Trolox/100 g DW.

Statistical analysis

The data are presented are mean values (n = 3) ± standard deviation. The data were subjected to analysis of variance (ANOVA), with a 95 % confidence level. Pearson's correlation analysis was performed to corroborate the relationships between bioactive compounds (polyphenols and carotenoids) and antioxidant activity, with a 95 % confidence level. The software used was Statgraphics Centurion version 16.1.18 (Statgraphics.Net, Madrid, Spain).

RESULTS AND DISCUSSION

Characterization of the phenolic composition

Guayusa polyphenols were identified by HPLC-DAD-ESI-MS^n methodology (Table 1) and quantification of individual phenolic compounds was carried out in HPLC-DAD. Besides, the traditional method of analysis of TPC was also carried out in the samples using Folin–Ciocalteu reagent (Table 2).

With regard to the detection of individual phenolics from *I. guayusa*, a characteristic chromatogram for these samples is shown in Figure 1.

The characteristic UV-Vis spectra, order of elution (Rt), parent ions (MS^-), along with the MS2 spectra provide confirmation to discriminate between the different phenolic acids and flavonols.

A total of 14 phenolic compounds were detected of which nine compounds corresponded to hydroxycinnamic acids and derivatives (peaks **1-7**, **12** and **14**) and 5 compounds were flavonoids, concretely flavonols (peaks **8 - 11** and **13**). It is important to emphasize that the order of elution observed was the same as previously described in Bravo et al.[20], with the exception of the 3,4-dicaffeoylquinic acid, in yerba mate leaves.

Caffeoyl derivatives

In relation to the caffeoyl quinic isomers detected, they are two compounds (peaks **1** and **5**) with similar mass spectra, with a $[M]^-$ ions at *m/z* 353 and 707 in the ESI- mode, and fragment ions at m/z 191 (base peak) and 179 (Table 1). The Rt and MS^n spectra of compound **5** was identical to the standard chlorogenic acid, which corresponds to caffeic acid esterified to quinic acid (5-*O*-caffeoyl quinic acid). Fragment ions at *m*/*z* 191 (base peak) and a weak 179 fragment (< 5 %), would correspond to deprotonated 5-

O-caffeoyl quinic acid and caffeic acid fragments, respectively, while *m/z* 707 mass was a dimeric adduct of the caffeoylquinic acid molecules. The other compound, peak **1**, would correspond to isomers of chlorogenic acid, concretely 3-*O*-caffeoyl quinic acid (neochlorogenic acid), where base peak *m/z* 191 and relatively intense secondary ion at *m/z* 179 (~50 %), according to Clifford et al.[21]

On the other hand, two compounds, **12** and **14**, with a $[M]^-$ ion at *m/z* 515 (Table 1) indicative of dicaffeoyl quinic acids, were also detected. Based on the MS and MS2 spectra and relative abundance, as well as the MS3 result of the base peak in the MS2 fragmentation[21], were identified as 3,5-dicaffeoylquinic acid (isochlorogenic acid) with MS2 at *m/z* 191, and 4,5-dicaffeoylquinic acid with MS2 at *m/z* 173, respectively (Table 1).

Apart from the major peaks, other compounds were identified as caffeoyl-hexoses (peaks **2, 3** and **4**) as suggested from the deprotonated molecular ion *m/z* of 341, the possible loss of caffeic acid, *m/z* = 162 (can be confused with the loss of hexose), and confirmed by the MS2 base peak at *m/z* of 179 (> 60 %), with an additional 135 m/z fragment.[21]

Other hydroxycinnamoyl quinic isomer

A compound at 20.7 min (compound **7**) showed the MS spectra of a 3-feruloylquinic acid, with a mass of 367 and a molecular intact ion at *m/z* of 193 (51 %) and 173 (0.8 %). Ion at *m/z* 193 corresponds to ferulic acid while ion at *m/z* 173 is dehydrated quinic acid.

Flavonols

Five peaks (compounds **8 - 11** and **13**) had MS spectra compatible with a flavonols. As described with the compound 5, the Rt and MS spectra of compounds **8** and **9** were

identical to the standard rutin, which corresponds to quercetin 3-*O*-rutinoside, showing a molecular ion m/z of 609 and fragments at m/z 301 (100 %) and 271. On the other hand, the peaks **10** and **11** were identified as quercetin 3-*O*-hexose by presenting a $[M]^-$ ion at *m/z* 463 and characteristic fragment ions at *m/z* 301 (100 %), according also with order of elution showed by Schieber et al.[19] for flavonols. Finally, peak **13** had $[M]^-$ ion at *m/z* 447 and a fragment at *m/z* 285 (100 %) in the negative mode, which is consistent with a kaempferol-3-*O*-hexose.

The different compounds identified have been previously described as typically constitute the phenolic fraction of the leaves of teas[22], mate (*I. paraguariensis*) [20,23] and other seven *Ilex* species (*I. brevicuspis*, *I. theezans*, *I. microdonta*, *I. dumosa* var. *dumosa*, *I. taubertiana*, *I. pseudobuxus*, *I. integerrima* and *I. argentina*).[5]

In relation to green leaves, quantitative analysis indicated that the hydroxycinnamic acid derivatives were the major constituents of the phenolic fraction (85% of the total phenolic) (Table 2). Caffeic acid was the main cinnamate, with mono- and dicaffeoyl quinic acid isomers accounting for approximately 75% and 90% of the total phenolic and hydroxycinnamic acids content, respectively. Bravo et al.[20] and Filip et al.[5] also described the hydroxycinnamic acid isomers as the principal constituents of the phenolic fraction of mate and seven *Ilex* species, respectively. Meanwhile, chlorogenic acid with 24.10 mg/g DW stood out as the most abundant phenolic compound. This concentration is similar or higher to that reported for the mate (21 – 28 mg/g)[5,24] and for seven *Ilex* species (0.42 – 9.15 mg/g)[5] and for tea (black tea= 0.5 mg/g; green tea= 0.2 mg/g)[25] but lower to that described for green coffee, a major source of chlorogenic acid in nature (50 – 120 mg/g).[26] Regarding their biological effects, numerous reports about chlorogenic acids focused on their antioxidant, anti-inflammatory, and anti-obesity activities, cancer chemoprevention, and a reduction in the cardiometabolic problems,

type 2 diabetes and Alzheimer's disease.[27] Chlorogenic acids are major constituents of plants, such as coffee beans[27], yerba mate[23] and other *Ilex* spp.[5]

In contrast, the flavonol fractions studied in tea and yerba mate, were described as rich in aglycones and glycosides.[5] The flavonols in guayusa were glycosides (Table 2). The total concentration of flavonols was around 11 mg/g DW. This concentration is around 2, 20 and 28 times higher than the previously described for yerba mate by Bravo et al.[20] (around 5 mg/g DW), other *Ilex* spp. by Filip et al.[4]5(around 0.5 mg/g), and for tea by Peterson et al.[28] (around 0.4 mg/g DW), respectively. Quercetin-3-*O*-hexose was the most abundant flavonol glycoside. Biological studies suggested that quercetin-3-*O*-hexose may have an antiviral, antibacterial, anticarcinogenic and anti-inflammatory effects.[29] Quercetin is also the main representative of the flavonols in tea varieties. [28]

The industrial processing could modify the profile and concentration of the phenolic fraction of guayusa. Thus, in the present study also the polyphenol composition of guayusa was evaluated after being subjected to industrial processes of blanching and fermenting. As shown in Table 2, both industrial processes did not modify the phenolic profile of guayusa. In relation with the concentration of these compounds, the processed guayusa showed significant differences with respect to the green leaves, with an increase in the content of polyphenols (except feruloyl-quinic acid) in the blanched guayusa and with a reduction in the content of phenolic compounds (except dicaffeoyl-quinic acid isomers) in the fermented guayusa (Table 2). During the blanching process, the temperature and humidity conditions as well as the fast cooling step would produce cell disruption which would lead to the subsequent release of polyphenols. This fact, together with the inactivation of enzymes involving phenolic oxidation, would contribute to a more efficient extraction of polyphenols from the blanched leaves and therefore, it would explain the high content of polyphenols observed in the guayusa

subjected to blanching. On the other hand, polyphenol concentrations during fermentation may decrease due to their diffusion out of their storage cells and further oxidation (non-enzymatic and/or catalysed by polyphenol oxidase and peroxidase) and complexation into high molecular mass. The results obtained in the blanched guayusa are in line with the reported previously in processed and commercial mate by Isolabella et al.[24] and Anesini et al.[30], respectively, while the results observed in fermented guayusa are agreed with the data described in black tea by Finger[31] and Sang.[32] On the other hand, as in green leaves, chlorogenic acid was the major phenolic compound found in the blanched guayusa while in the fermented guayusa 3,5-dicaffeoylquinic acid (isochlorogenic acid) was the most abundant compound. The lowest 3,5-dicaffeoylquinic acid content in the fermented guayusa could be due to the oxidation (non-enzymatic and/or enzymatic) of this compound during fermentation process.

Finally, green leaves showed a TPC of 54.86 mg GAE/ g DW. This content is similar to that reported by Pardau[4] in green guayusa (54.92 mg GAE/ g), and higher to that reported by Moraes de Souza et al.[33] in some processed tea and herbal infusions (ranging 0 – 46 mg GAE/g), by Valerga et al.[34] for fresh leaves of mate (4.15 mg GAE/g) and by Oh et al.[35] for various leafy herbal teas. However, our results are lower to that described for green and black tea, water extracts, (around 82 mg GAE/g) by Oh et al.[35] In relation to processed guayusa, blanching led to a significant increase (48.5%) in the TPC of guayusa in comparison to the green leaves, but fermentation did not show significant differences with the non-treated leaves. The blanched guayusa showed the highest TPC, being this content (107 mg GAE/g) higher than observed in processed mate[34] and green and black tea, water extracts.[35]

Carotenoids

With regard to carotenoids, a total of 5 carotenoid compounds were quantified in green leaves and guayusa processed: α and β-carotene, lutein and violaxanthin+neoxanthin (Table 3). The total content of carotenoids evaluated as the sum of the content of individual pigments did not show significant differences between green leaves and guayusa processed, differences were only significant for blanched and fermented guayusa (Table 3). The total of carotenoids ranged from 286.65 to 468.71 μg/g DW, which was in good agreement with the results reported by Ravichandran[36] in tea leaves. On the other hand, lutein was the main carotenoid in the green leaves, which agreed with the results reported by other authors in green leafy vegetables.[37,38] This carotenoid plays important roles in human health, such as the reduction of risk of diseases as cancer, cataracts and age related macular degeneration.[38-40] The contents of β-carotene and lutein were in the range reported in teas[36,37,40] while the concentrations of α-carotene and violaxanthin were higher as compared to other reported data.[36]

Being highly unsaturated, carotenoids are susceptible to modifications (i.e., isomerization and oxidation) during processing.[38,41] Therefore, it was evaluated how industrial processes, particularly blanching and fermentation, could modify its concentration. Thus, the blanched guayusa exhibited a higher content of β-carotene and lutein than green leaves (305.39 and 141.52 %, respectively), but lower concentration of α-carotene and violaxanthin+neoxanthin (55.27 and 22.38%, respetively) (Table 3). In this sense, it appears that blanching of guayusa leaves is important to retain the levels of these health-promoting carotenoids.

Similarly, to phenolic compounds, the fermented guayusa showed the lowest levels of carotenoids, except β-carotene (Table 3). The fermented guayusa, exhibited lower contents of α-carotene and violaxanthin+neoxanthin than green leaves (p<0.05), but it did not show significant differences in the concentrations of β-carotene and lutein

(Table 3). Silveira et al.[39] described in the mate that the increase of the temperature of the water for infusion favored the transfer of lutein. This could explain the high content of lutein in the blanched guayusa, because this industrial process proceeds at a high temperature water.

On the other hand, the major degradation of the carotenoids α-carotene and violaxanthin+neoxanthin in the fermented guayusa could be attributed to the oxidative degradation (enzymatic or non-enzymatic), principal cause of losses of carotenoids.[36,38,41] In addition, the oxidative enzymes are inactivated during blanching[41] which would explain that despite of the heat treatment employed in this process, the destruction of α-carotene and violaxanthin+neoxanthin was lower in the blanched guayusa than in fermented guayusa. With regard to violaxanthin+neoxanthin, it was the most labile carotenoid to the industrial treatments, with percentages of degradation of the 77.6% and 92.5% in the blanched guayusa and fermented guayusa, respectively. These results are in accordance with the results reported by other authors in green vegetables.[38,42] The changes observed in the content of carotenoid during guayusa processing could origin the production of various carotenoid-derived aroma compounds that would contribute positively to the organoleptic characteristic of guayusa, which was in agreement with that described by other authors in the manufactured tea.[36,43]

Antioxidant capacity

Numerous studies have highlighted the antioxidant capacity of polyphenols and carotenoids. [7,38] Antioxidant properties of the green leaves and processed guayusa were evaluated on the basis of measuring scavenging activity for DPPH radicals and the inhibition of peroxyl radical induced oxidations by antioxidants by ORAC method. The results for the antioxidant capacity are shown in Table 4. The values obtained for the

green leaves in the DPPH assay (32.98 mM Trolox/100 g DW) and ORAC assay (154.03 mM Trolox/100 g DW) are higher and similar to the values reported by Pardau[4] in green guayusa in DPPH (11.51 mM Trolox/100 g) and ORAC (156.75 mM Trolox/100) assays, respectively. In addition, the values obtained are in accordance with the results reported by Chandra and De Mejía[44] and Vieira et al.[45] in beverages with high antioxidant capacity as mate and green teas, respectively. As shown with the previous results, during the industrial processing stages (i.e., blanching and fermenting) were observed some changes in the concentrations of bioactive compounds (polyphenols and carotenoids) of guayusa, which were reflected in significant differences in the values of antioxidant capacity of green leaves and processed guayusa (Table 4). This result is in line with that reported by other authors[24,46] in mate and commercial mate. In addition, the guayusa with major contents of polyphenols and carotenoids, green leaves and blanched guayusa, exhibited the highest antioxidant capacity. The polyphenol and carotenoid contents showed a positive and direct correlation with the antioxidant capacity (DPPH and ORAC), especially with ORAC (Table 5). Furthermore, the concentrations of phenolic compounds was higher than carotenoids (Table 2 and 3) which could suggest that the high antioxidant capacity observed in guayusa might be attributed mainly to the polyphenols; and particularly mono- and dicaffeoylquinic acid isomers present in major quantities in the guayusa tested. Polyphenols are also considered the main responsible for other foods such as tea,[28] red wine,[47] cocoa[47] and mate,[20] among others.

CONCLUSIONS

This work reports a complete study on the phenolic and carotenoid composition of *I. guayusa*. Antioxidant capacity is comparable to that of beverages known for their high

antioxidant power, like tea or mate. On the other hand, the composition of guayusa is clearly influenced by processing. In general, blanching shows higher content of polyphenols and some carotenoids (β-carotene and lutein) while fermenting reduces the concentration of these bioactive compounds as well as the antioxidant capacity. In addition, the results suggest that regular consumption of guayusa would significantly contribute to the antioxidant intake, providing polyphenols and carotenoids with biological effects potentially beneficial to human health. Therefore, considering the increased consumption of functional foods, the guayusa, especially blanched guayusa, could be used as a supplement in the human diet and as an ingredient with functional components. Future studies are necessary to evaluate the presence and bioavailability of bioactive compounds of guayusa in greater detail.

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REFERENCES

1. Kapp RW, Mendes O, Roy S, McQuate RS and Kraska R, General and genetic toxicology of guayusa concentrate (*Ilex guayusa*). *Int J Toxicol* **35**: 222-242 (2016).

2. Lewis WH, Kennelly EJ, Bass GN, Wedner HJ, Elvin-Lewis MP and Fast D, Ritualistic use of the holly *Ilex guayusa* by Amazonian Jivaro Indians. *J Ethnopharmacol* **33**: 25-30 (1991).

3. Bennett BC, Hallucinogenic plants of the Shuar and related indigenous groups in Amazonian Ecuador and Peru. *Brittonia* **44**: 483-493 (1992).

4. Pardau MD, Antioxidant activity and nitric oxide scavenging abilities of *Ilex guayusa* compared with *Camellia sinensis* and *Asphalathus linearis* teas. MSc Thesis. University of Pretoria. (2016)

5. Filip R, López P, Giberti G, Coussio J and Ferraro G, Phenolic compounds in seven South American *Ilex* species. *Fitoterapia* **72**: 774-778 (2001).

6. Zielinski AAF, Haminiuk CWI, Alberti A, Nogueira A, Demiate IM and Granato D, A comparative study of the phenolic compounds and the in vitro antioxidant activity of different Brazilian teas using multivariate statistical techniques. *Food Res Int* **60**: 246-254 (2014).

7. Vasantha Rupasinghe HP, Nair SVG and Robinson RA, Chemopreventive properties of fruit phenolic compounds and their possible mode of actions. *Studies in Natural Products Chemistry*: 229-266 (2014).

8. Krinsky NI and Johnson EJ, Carotenoid actions and their relation to health and disease. *Mol Aspects Med* **26**: 459-516 (2005).

9. Navarro M and Núñez, O Liquid Chromatography-Mass Spectrometry in the analysis and characterization of proanthocyanidins in natural products. In Proanthocyanidins:

Food Sources, Antioxidant Properties and Health Benefits; Nova Science Publishers: Hauppauge, New York, USA. Chapter 1. (2015).

10. Stinco CM, Benítez-González AM, Hernanz D, Vicario IM and Meléndez-Martínez AJ, Development and validation of a rapid resolution liquid chromatography method for the screening of dietary plant isoprenoids: Carotenoids, tocopherols and chlorophylls. *J Chromatogr A* **1370**: 162-170 (2014).

11. García-Ruiz A, Girones-Vilaplana A, León P, Moreno DA, Stinco CM, Meléndez-Martínez AJ and Ruales J, Banana passion fruit (*Passiflora mollissima* (Kunth) L.H. Bailey): microencapsulation, phytochemical composition and antioxidant capacity. *Molecules* **22**: 85 (2017).

12. Rahman MS and Perera CO, Drying and food preservation. In Handbook of book preservation (Second Edition). Ed.: M. Shafiur Rahman, CRC Press, New York. Chapter 18. (2007)

13. Dudonné S, Dubé P, Anhê FF, Pilon G, Marette A, Lemire M, Harris C, Dewailly E and Desjardins Y, Comprehensive analysis of phenolic compounds and abscisic acid profiles of twelve native Canadian berries. *J Food Comp Anal* **44:** 214-224 (2015).

14. Tierno R, Hornero-Méndez D, Gallardo-Guerrero L, López-Pardo R and de Galarreta JIR, Effect of boiling on the total phenolic, anthocyanin and carotenoid concentrations of potato tubers from selected cultivars and introgressed breeding lines from native potato species. *J Food Comp Anal* **41**: 58-65 (2015).

15. Meléndez-Martínez AJ, Britton G, Vicario IM and Heredia FJ, Relationship between the colour and the chemical structure of carotenoid pigments. *Food Chem* **10**: 1145-1150 (2006).

16. Slinkard K and Singleton VL, Total phenol analysis: Automation and comparison with manual methods. *Am J Enol Vitic* **28**: 49-55 (1997).

17. Gironés-Vilaplana A, Baenas N, Villaño D, Speisky H, García-Viguera C and Moreno DA, Evaluation of Latin-American fruits rich in phytochemicals with biological effects. *J Funct Foods* **7**: 599-608 (2014).

18. Mena P, García-Viguera C, Navarro-Rico J, Moreno DA, Bartual J, Saura D and Martí N, Phytochemical characterisation for industrial use of pomegranate (*Punica granatum* L.) cultivars grown in Spain. *J Sci Food Agric* **91**: 1893–1906 (2011).

19. Ou B, Hampsch-Woodill M and Prior RL, development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J Agric Food Chem* **49**: 4619–4626 (2001).

20. Bravo L, Goya L and Lecumberri E, LC/MS characterization of phenolic constituents of mate (*Ilex paraguariensis*, St. Hil.) and its antioxidant activity compared to commonly consumed beverages. *Food Res Int* **40**: 393–405 (2007).

21. Clifford MN, Johnston KL, Knight S and Kuhnert N, Hierarchical scheme for LC-MS^n identification of chlorogenic acids. *J Agric Food Chem* **51**: 2900-2911 (2003).

22. Bastos DH, Saldanha LA, Catharino RR, Sawaya AC, Cunha IB, Carvalho PO and Eberlin MN, Phenolic antioxidants identified by ESI-MS from Yerba maté (*Ilex paraguariensis*) and green tea (*Camelia sinensis*) extracts. *Molecules* **12**: 423-432 (2007).

23. Peres RG, Tonin FG, Tavares MFM and Rodriguez-Amaya DB, HPLC-DAD-ESI/MS identification and quantification of phenolic compounds in *Ilex paraguariensis*

beverages and on-line evaluation of individual antioxidant activity. *Molecules* **18**: 3859-3871 (2013).

24. Isolabella S, Cogoi L, López P, Anesini C, Ferraro G and Filip R, Study of the bioactive compounds variation during yerba mate (*Ilex paraguariensis*) processing. *Food Chem* **122**: 695 – 699 (2010).

25. Marques V and Farah A, Chlorogenic acids and related compounds in medicinal plants and infusions. *Food Chem* **113**: 1370–1376 (2009).

26. Farah A and Donangelo CM, Phenolic compounds in coffee. *Braz J Plant Physiol* **18**: 23–36 (2006).

27. Farah A, Monteiro M, Donangelo CM and Lafay S, Chlorogenic acids from green coffee extract are highly bioavailable in humans. *J Nutr* **138**: 2309-2315 (2008).

28. Peterson J, Dwyer J, Bhagwat S, Haytowitz D, Holden J, Eldridge AL, Beecher G and Aladesanmi J, Major flavonoids in dry tea. *J Food Compost Anal* **18**: 487–501 (2005).

29. Chirumbolo S, The role of quercetin, flavonols and flavones in modulating inflammatory cell function. *Inflamm Allergy Drug Targets* **9**: 263-285 (2010).

30. Anesini C, Turner S, Cogoi L and Filip R, Study of the participation of caffeine and polyphenols on the overall antioxidant activity of mate (*Ilex paraguariensis*). *LWT - Food Sci. Tech.* **45**: 299 – 304 (2012).

31. Finger A, *In-vitro* studies on the effect of polyphenol oxidase and peroxidase on the formation of polyphenolic black tea constituents. *J Sci Food Agric* **66**: 293-305 (1994).

32. Sang S, Tea: chemistry and processing. *Reference Module in Food Science, Encyclopedia of Food and Health,* 268 – 272 (2016).

33. Moraes-de-Souza RA, Oldoni TLC, Regitano-d'Arce MAB and Alencar SM, Antioxidant activity and phenolic composition of herbal infusions consumed in Brazil. *Ciên. Tecnol Aliment* **6**: 41-47 (2008).

34. Valerga J, Reta M and Lanari MC, Polyphenol input to the antioxidant activity of yerba mate (*Ilex paraguariensis*) extracts. *LWT - Food Sci Tech* **45**: 28-35 (2012).

35. Oh J, Jo H, Cho AR, Kim S-J and Han J, Antioxidant and antimicrobial activities of various leafy herbal teas. *Food Control* **31**: 403-409 (2013).

36. Ravichandran R, Carotenoid composition, distribution and degradation to flavor volatiles during black tea manufacture and the effect of carotenoid supplementation on tea quality and aroma. *Food Chem* **78**: 23 – 28 (2002).

37. Loranty A, Rembiałkowska E, Rosa EAS and Bennett RN, Identification, quantification and availability of carotenoids and chlorophylls in fruit, herb and medicinal teas. *J Food Comp Ana.* **23**: 432-441 (2010).

38. Rodríguez-Amaya DB, Rodríguez EB and Amaya-Farfán J, Advances in food carotenoid research: chemical and technological aspects, implications in human health. *Malays J Nutr* **12**: 101 – 121 (2006).

39. Silveira TFFD, Meinhart AD, Coutinho JP, de Souza TCL, Cunha ECE, de Moraes MR and Godoy HT, Content of lutein in aqueous extracts of yerba mate (*Ilex paraguariensis* St. Hil). *Food Res Int* **82**: 165 – 171 (2016).

40. Heo J-Y, Kim S, Kang J-H and Moon B, Determination of lutein from green tea and green tea by-products using accelerated solvent extraction and UPLC. *J Food Sci* **79**: C816-C821 (2014).

41. Rodríguez-Amaya DB, Changes in carotenoids during processing and storage of foods. *Arch Latinoam Nutr* **49**: 38S-47S (1999).

42. De Sá MC and Rodríguez-Amaya DB, Carotenoid composition of cooked green vegetables from restaurants. *Food Chem* **83**: 595-600 (2003).

43. Ho CH, Zheng X and Li S, Tea aroma formation. *Food Sci Human Wellness* **4**: 9–27 (2015).

44. Chandra S and De Mejía EG, Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of Ardisia compressa in comparison to mate (*llex paraguariensis*) and green (*Camellia sinensis*) teas. *J Agr Food Chem* **52**: 3583-3589 (2004).

45. Vieira MA, Maraschin M, Pagliosa CM, Podestá R, de Simas KN, Rockenbach II, Amboni RDDMC and Amante ER, Phenolic acids and methylxanthines composition and antioxidant properties of mate (*Ilex paraguariensis*) residue. *J Food Sci* **75**: C280-C285 (2010).

46. Turner S, Cogoi L, Isolabella S, Filip R and Anesini C, Evaluation of the antioxidant activity and polyphenols content of *Ilex paraguariensis* (maté) during industrialization. *Adv. J Food Sci Technol* **3**: 23–30 (2011).

47. Dreosti IE, Antioxidant polyphenols in tea, cocoa, and wine. *Nutrition* **16**: 692 – 694 (2000).

FIGURE CAPTIONS

Figure 1. Typical chromatogram of guayusa, registered at 360 nm (A) and 330 nm (B) for the identification and quantification of phenolic compounds in the samples. For compound assignment see Table 1.

TABLES

Table 1. Tentative identification of phenolics in guayusa samples by HPLC–DAD–ESI/MS^n

Peak	Rt (min)	M^-	MS2 fragments	Phenolic Compounds
1	6.0	707*, 353	191 (100), 179 (42.6)**	3-*O*-CQA (Neochlorogenic acid)
2	6.6	341	179 (63.0), 135 (10.6)	Caffeoyl-hexose
3	8.2	341	179 (100), 135 (10.4)	Caffeoyl-hexose
4	9.6	341	179 (78.9), 135 (4.9)	Caffeoyl-hexose
5	10.6	353	191 (100), 179 (4.7)	5-*O*-CQA (Chlorogenic acid)
6	12.5	707*, 353	191 (100), 179 (3.2)	5-*O*-CQA isomer
7	20.7	367	193 (51.3), 173 (0.8)	3-Feruloylquinic acid
8	27.3	609	301 (100), 271 (23.1)	Quercetin-3-*O*-rutinoside (Rutin)
9	28.5	609	301 (100), 271 (15.2)	Rutin Isomer
10	32.5	463	301 (100)	Quercetin-3-*O*-hexose
11	34.3	463	301 (100)	Quercetin-3-*O*-hexose
12	36.1	515	353 (100), 191 (9.7)†, 179 (1.3)	3,5-Dicaffeoylquinic acid (Isochlorogenic acid)
13	42.8	447	285 (100)	Kaempferol-3-*O*-hexose
14	43.7	1031*, 515	353 (61.4), 173 (10.43)†	3,4-Dicaffeoylquinic acid

Rt: retention time, * Dimeric adduct, **Relative abundance (software ca. %); †Base Peak in MS2 (MS3 100% Rel. abun.). CQA (Caffeoylquinic acid)

Table 2. Phenolic composition in green leaves and processed guayusa

		Concentration (mg/g DW)		
		Green leaves	**Blanched guayusa**	**Fermented guayusa**
	Total Phenolics Content*	$54.86^{b} \pm 3.09$	$106.62^{a} \pm 4.41$	$59.47^{b} \pm 1.15$
Peak	***Compounds***			
1	3-*O*-CQA (Neochlorogenic acid)	$7.93^{b} \pm 0.34$	$11.30^{a} \pm 1.06$	$2.55^{c} \pm 0.25$
2	Caffeoyl-hexose	$1.10^{b} \pm 0.05$	$1.31^{a} \pm 0.14$	$0.12^{c} \pm 0.01$
3	Caffeoyl-hexose	$4.98^{a} \pm 0.73$	$5.17^{a} \pm 0.17$	$1.29^{b} \pm 0.21$
4	Caffeoyl-hexose	$0.76^{b} \pm 0.09$	$1.71^{a} \pm 0.18$	$0.21^{c} \pm 0.10$
5	5-*O*-CQA (Chlorogenic acid)	$24.10^{a} \pm 1.59$	$26.53^{a} \pm 0.84$	$7.53^{b} \pm 0.09$
6	5-*O*-CQA† isomer	< Q.L.	< Q.L.	< Q.L.
7	3-Feruloylquinic acid	$0.60^{a} \pm 0.05$	$0.58^{a} \pm 0.02$	$0.21^{b} \pm 0.01$
8	Quercetin-3-*O*-rutinoside	$0.60^{b} \pm 0.00$	$0.70^{a} \pm 0.02$	$0.32^{c} \pm 0.02$
9	Quercetin-3-*O*-rutinoside	$0.88^{b} \pm 0.02$	$0.96^{a} \pm 0.04$	$0.48^{c} \pm 0.02$
10	Quercetin-3-*O*-hexose	$2.64^{b} \pm 0.19$	$3.35^{a} \pm 0.33$	$1.54^{c} \pm 0.05$
11	Quercetin-3-*O*-hexose	$5.72^{b} \pm 0.66$	$5.96^{a} \pm 0.18$	$2.88^{b} \pm 0.17$
12	3,5-Dicaffeoylquinic acid (Isochlorogenic acid)	$16.61^{b} \pm 0.20$	$23.34^{a} \pm 0.07$	$17.55^{b} \pm 1.25$
13	Kaempferol-3-*O*-glucoside	$1.02^{b} \pm 0.12$	$1.56^{a} \pm 0.12$	$0.79^{c} \pm 0.13$
14	3,4- Dicaffeoylquinic acid	$3.28^{b} \pm 0.05$	$4.74^{a} \pm 0.32$	$4.53^{a} \pm 0.60$

*Total phenolics content, by Slinkard and Singleton method, is expressed as mg of gallic acid equivalents per g.

a – c Mean values with different letter on the right in the same row indicate statistically significant differences among the three treatments ($p<0.05$).

†CQA (Caffeoylquinic acid) < Q.L. (quantitation limit).

Table 3. Carotenoid composition in green leaves and processed guayusa

	Concentration (μg/g DW)		
	Green leaves	**Blanched guayusa**	**Fermented guayusa**
Compounds			
α-Carotene	$113.12^{a} \pm 19.81$	$62.52^{b} \pm 15.59$	$46.76^{b} \pm 2.00$
β-Carotene	$37.85^{b} \pm 5.09$	$115.59^{a} \pm 27.46$	$58.34^{b} \pm 0.41$
Lutein	$188.19^{ab} \pm 46.69$	$266.33^{a} \pm 65.38$	$173.38^{b} \pm 3.65$
Violaxanthin+Neoxanthin	$108.46^{a} \pm 25.25$	$24.27^{b} \pm 2.06$	$8.17^{b} \pm 0.34$
Total carotenoids	$447.62^{ab} \pm 96.84$	$468.71^{a} \pm 110.49$	$286.65^{b} \pm 6.4$

a – b Mean values with different letter on the right in the same row indicate statistically significant differences among the three treatments ($p<0.05$).

Table 4. Antioxidant capacity in green leaves and processed guayusa

	Antioxidant capacity (mmol Trolox/100g DW)		
	Green leaves	**Blanched guayusa**	**Fermented guayusa**
DPPH	$33.0^{b} \pm 1.4$	$35.4^{a} \pm 1.0$	$19.7^{c} \pm 0.2$
ORAC	$154.0^{a} \pm 20.4$	$111.3^{b} \pm 25.0$	$76.0^{c} \pm 5.3$

a – c Mean values with different letter on the right in the same row indicate statistically significant differences among the three treatments ($p<0.05$).

Table 5. Pearson's correlation coefficients (*r*) between bioactive compounds (flavonoids and carotenoids) green leaves and processed guayusa and its antioxidant capacity (DPPH and ORAC).

	Samples		
	Green leaves	**Blanched guayusa**	**Fermented guayusa**
DPPH	0.73	0.98	0.80
ORAC	0.99	1.00	1.00

Significant at $p < 0.05$

FIGURES